REMARKS

Claims 2-3, 6, 8-12, 15, 46-48, 50, 76-83, 85-94, and 96-104 are pending in this application. These claims have been cancelled herein without prejudice or disclaimer of the subject matter contained therein. These claims have generally been rewritten as new claims 106-136. In particular, independent claim 2 has been rewritten as new claim 106, independent claim 76 has been rewritten as new claim 118, and independent claim 87 has been rewritten as new claim 127. Support for these new claims can be found, *inter alia*, in the claims as originally filed, in the support previously indicated for the previously pending claims, and throughout the specification. Accordingly, Applicant submits that no new matter has been added by these new claims.

Therefore, after entry of this amendment, claims 106-136 will be pending in the application.

Applicant thanks Examiner Anne Marie Falk, Ph.D. for her time for participating in a telephonic interview on March 7, 2006 with legal representatives Ann-Louise Kerner, Ph.D. and Alison Corkery; inventor, Dr. Bruestle; and European counsel for the inventor, Dr. Martin Grund. Dr. Grund did not participate in the interview.

During the interview, the outstanding written description rejection under 35 U.S.C. § 112, first paragraph was discussed, as well as whether there was support in the specification for language proposed in draft claims. The outstanding rejection under 35 U.S.C. § 102 over U.S. Patent No. 5,980,885 (Weiss *et al.*) was also discussed. Applicant's representatives and Dr. Bruestle pointed out some differences in cells derived from embryonic stem cells compared to neural cells. The Examiner indicated that for a product-by-process claim, only structural limitations would be considered, and accordingly, a distinguishing property would need to be in the claim.

The outstanding rejections will be addressed separately below.

I. The New Claims Corresponding to Claims 2, 3, 6, 8-12, 15, 46-48, 50, 76-83, 85-94, and 96-104 as Previously Filed Do Not Contain New Matter

Claims 2, 3, 6, 8-12, 15, 46-48, 50, 76-83, 85-94, and 96-104 were rejected under 35 U.S.C. § 112, first paragraph, as allegedly failing to comply with the written description requirement. These claims have been cancelled herein without prejudice or disclaimer of the subject matter contained therein. These claims generally correspond to new claims 106-136.

The Office Action states that the specification does not provide support for the claim language of a cell composition comprising "about 100% isolated neural cells and neural precursor cells." (Office Action, page 4) Applicant respectfully disagrees.

However, in order to expedite prosecution, the claims have been rewritten to indicate, using claim 106 as an example, a cell composition consisting essentially of embryonic stem cell-derived neural precursor cells, and neuronal or glial cells derived from the embryonic stem cell-derived neural precursor cells, as indicated in the specific claim language of the individual claims.

M.P.E.P. 2163.05 states that to "comply with the written description requirement of 35 U.S.C. 112, para. 1, or to be entitled to an earlier priority date or filing date under 35 U.S.C. 119, 120, or 365(c), each claim limitation must be expressly, implicitly, or inherently supported in the originally filed disclosure." Furthermore, M.P.E.P. 2163(II)(A)(2) states that the "analysis of whether the specification complies with the written description requirement calls for the examiner to compare the scope of the claim with the scope of the description to determine whether applicant has demonstrated possession of the claimed invention."

The specification provides evidence that Applicant was in possession of such of cell compositions. This fact is evidenced in the specification at, *inter alia*, page 24, lines 31-38, which show data indicating the presence of neural antigens for neural precursor cells as well as for neurons, astrocytes, and cells with oligodendroglial morphology in a

cell composition according to many of the presently claimed embodiments of the invention. In addition, the specification provides evidence that the cell compositions contain embryonic stem cell-derived neural precursor cells, and neuronal or glial cells derived from the embryonic stem cell-derived neural precursor cells, when discussing differentiation of the precursor cells as described in the specification at, *inter alia*, Fig. 1 (as described at page 10, lines 13-26); Fig. 2 (as described at page 10, line 29 to page 11, line 5); Fig. 3 (as described at page 11, lines 7-19); Fig. 5 (as described at page 12, line 4 to page 13, line 7); FIG. 7 (as described at page 13, lines 18-29); page 15, line 39 to page 16, line 5; page 16, line 23 to page 17, line 4; page 17, line 33 to page 18, line 9; page 23, line 30 to page 24, line 2; page 24, line 20 to page 25, line 18; and page 26, lines 32-33. These same sections provide support for the language of the other independent claims as well.

Furthermore, as indicated in the Second Declaration of Dr. Oliver Bruestle Under 37 C.F.R. § 1.132 (provided herewith), based on the disclosure provided in the specification (of which the above examples are a part), one of skill in the art would clearly be aware that cell compositions consisting essentially of different proportions of embryonic stem cell-derived neural precursor cells, and neuronal or glial cells derived from the embryonic stem cell-derived neural precursor cells, would and could easily be obtained based on the disclosure in the specification. For example, one of skill in the art would already be aware that depending on the amount of time allowed and the growth factors used for proliferation or differentiation, neural precursor cells would eventually give rise to neuronal or glial cells and the relative proportion of embryonic stem cell-derived neural precursor cells, and neuronal or glial cells derived from the embryonic stem cell-derived neural precursor cells, would change and evolve as the various types of cells proliferated and differentiated at different rates. In addition, based on the disclosure in the specification, one of skill in the art would know that these cell compositions consisted essentially of embryonic stem cell-derived neural precursor cells, and neuronal or glial cells that evolved or were derived from these embryonic stem cell-derived neural precursor cells. Thus, one of skill in the art would know that

Applicant was in possession of the claimed invention, and described how to make and use the claimed invention, using the information provided in the specification. This same information provides support for the language of the other independent claims as well.

Accordingly, Applicant submits that the new matter rejection with regard to the rejected claims is moot and that new claims 106-136 are adequately supported by the specification as filed. Applicant respectfully requests that this rejection be reconsidered and withdrawn.

II. New Claims Corresponding to Claims 2, 3, 6, 8-12, 15, 46-48, 50, 76-83, 85-94, and 96-104 as Previously Filed Do Not Fail to Comply with the Enablement Requirement

Claims 2, 3, 6, 8-12, 15, 46-48, 50, 76-83, 85-94, and 96-104 stand or were rejected under 35 U.S.C. § 112, first paragraph, for allegedly failing to comply with the enablement requirement. These claims have been cancelled herein without prejudice or disclaimer of the subject matter contained therein. These claims generally correspond to new claims 106-136.

A. Rejection regarding language "about 100% isolated neural cells and neural precursor cells"

The Office Action states that the section cited in Applicant's previous response does not describe a cell composition comprising "about 100% isolated neural cells and neural precursor cells." The Office Action further states that there is "nothing to suggest that these cell types make up 100% of the cells of the composition" and it appears "that the cell composition produced was not analyzed for the presence of other cell types."

M.P.E.P. § 2164.01 states

> Any analysis of whether a particular claim is supported by the disclosure in an application requires a determination of whether that disclosure, when filed, contained sufficient information regarding the subject matter of the claims as to

> enable one skilled in the pertinent art to make and use the claimed invention. . . . Accordingly, even though the statute does not use the term "undue experimentation," it has been interpreted to require that the claimed invention be enabled so that any person skilled in the art can make and use the invention without undue experimentation. . . .
>
> The fact that experimentation may be complex does not necessarily make it undue, if the art typically engages in such experimentation. (citations omitted)

Furthermore, M.P.E.P. § 2164.08 states

> Nevertheless, not everything necessary to practice the invention need be disclosed. In fact, what is well-known is best omitted. . . . . Further the scope of enablement must only bear a "reasonable correlation" to the scope of the claims. (Emphasis added) (citations omitted)

As discussed above, the specification provides evidence that Applicant was in possession of such cell compositions at the time the application was filed. This fact is evidenced in the specification at, *inter alia*, page 24, lines 31-38, which show data indicating the presence of neural antigens for neural precursor cells as well as for neurons, astrocytes, and cells with oligodendroglial morphology in a cell composition according to many of the presently claimed embodiments of the invention. In addition, the specification provides evidence that the cell compositions contain embryonic stem cell-derived neural precursor cells, and neuronal or glial cells derived from the embryonic stem cell-derived neural precursor cells, when discussing differentiation of the precursor cells as described in the specification at, *inter alia*, Fig. 1 (as described at page 10, lines 13-26); Fig. 2 (as described at page 10, line 29 to page 11, line 5); Fig. 3 (as described at page 11, lines 7-19); Fig. 5 (as described at page 12, line 4 to page 13, line 7); FIG. 7 (as described at page 13, lines 18-29); page 15, line 39 to page 16, line 5; page 16, line 23 to page 17, line 4; page 17, line 33 to page 18, line 9; page 23, line 30 to page 24, line 2; page 24, line 20 to page 25, line 18; and page 26, lines 32-33. These same sections provide support for the language of the other independent claims as well.

Furthermore, as additionally discussed above and in the Second Declaration of Dr. Oliver Bruestle submitted herewith, based on the disclosure provided in the specification (of which the above examples are a part), one of skill in the art would clearly be aware that cell compositions consisting essentially of different proportions of embryonic stem cell-derived neural precursor cells, and neuronal or glial cells derived from the embryonic stem cell-derived neural precursor cells, would and could easily be obtained based on the disclosure in the specification. For example, one of skill in the art would already be aware that depending on the amount of time allowed and the growth factors used for proliferation or differentiation, neural precursor cells would eventually give rise to neuronal or glial cells and the relative proportion of embryonic stem cell-derived neural precursor cells, and neuronal or glial cells derived from the embryonic stem cell-derived neural precursor cells, would change and evolve as the various types of cells proliferated and differentiated at different rates. In addition, based on the disclosure in the specification, one of skill in the art would know that these cell compositions consisted essentially of embryonic stem cell-derived neural precursor cells, and neuronal or glial cells that evolved or were derived from these embryonic stem cell-derived neural precursor cells. Thus, one of skill in the art would know that Applicant was in possession of the claimed invention, and described how to make and use the claimed invention, using the information provided in the specification. This same information provides support for the language of the other independent claims as well.

In addition, the Declaration of Dr. Oliver Bruestle under 37 C.F.R. § 1.132 ("First Declaration"), which was submitted September 5, 2003 (the unexecuted, unrevised version of which was "fully considered" by the Examiner, Advisory Action dated 10/03/2003, page 2) states in part that, because there are no unequivocal markers for defining neural precursor cells, Applicant applied "a cocktail of antibodies to markers of both immature (nestin and A2B5) and differentiated (beta-III-tubulin, GFAP, and 04) neural precursor cells" to show that "the cell compositions of the present invention contain more than 99% neural cells" (page 6, paragraph 17). Thus, this composition

included essentially only neural precursor cells and neuronal or glial cells derived therefrom.

Furthermore, Figure 5C and the accompanying description on page 12, lines 9-12 of the specification provide data showing that five-day-old neural spheres, derived from CJ7 ES cells, were manipulated to "have disintegrated and differentiated into neural cells." Also, Figure 5D shows "a double immunofluorescence analysis using antibodies to the neuronal antigen beta-III-tubulin (bright signal) and the neural precursor cell marker nestin (dark signal, arrows)" and further, that "**all cells depicted in this field express either of the two markers**" (page 12, lines 12-16) (emphasis added).

Therefore, because all cells expressed either a marker associated with a neural precursor cell or a glial cell (nestin is expressed in neural precursors and glial cells, see First Declaration, page 6, paragraph 15) or a marker associated with a neuronal cell, Applicant submits that this information, in addition to data in the declaration and evidence from the specification, shows that Applicant has indeed demonstrated that the claimed cell composition consists essentially of embryonic stem cell-derived neural precursor cells, and neuronal or glial cells derived from the embryonic stem cell-derived neural precursor cells (otherwise tumors or aberrant cell types would have been detected). This same evidence provides support for the enablement of the other independent claims as well.

The Office Action doubts the accuracy of this argument regarding Figure 5D because "a field of view under a microscope is not sufficient to describe an entire cell composition" and there is "nothing to suggest that the particular field of view shown in Figure 5D is representative of the entire cell composition." (Emphasis added) (Office Action, page 5)

However, M.P.E.P. § 2164.04 states that

> A specification disclosure which contains a teaching of the manner and process of making and using an invention in terms which correspond in scope to those used in describing and defining the subject matter sought to be patented must be taken as being in compliance with the enablement requirement of 35 U.S.C. 112, first paragraph, unless there is a reason to doubt the objective truth of the statements contained therein which must be relied on for enabling support. . . . As stated by the court, "it is incumbent upon the Patent Office, whenever a rejection on this basis is made, to explain *why* it doubts the truth or accuracy of any statement in a supporting disclosure and to back up assertions of its own with acceptable evidence or reasoning which is inconsistent with the contested statement. Otherwise there would be no need for the applicant to go to the trouble and expense of supporting his presumptively accurate disclosure." 439 F.2d at 224, 169 USPQ at 370. (Emphasis added)

Therefore, Applicant respectfully submits that without evidence or reasoning regarding reasons to doubt that the particular field of view shown in Figure 5D is representative of the entire cell composition, Applicant does not need to provide proof of multiple fields of view to indicate the results for the entire cell composition.

Thus, Applicant respectfully submits that the newly filed claims are enabled. Accordingly, Applicant respectfully requests that this rejection be reconsidered and withdrawn.

B. Rejection regarding species of embryonic stem cells

The Office Action also states that the skilled artisan would allegedly find it unpredictable whether protocols developed for the mouse would be appropriate for ES-like cells derived from other species, particularly non-mammalian species such as birds and fish. (Office Action, page 6)

Although Applicant does not necessarily agree with this determination, in order to expedite prosecution, the independent claims have been amended to state that the embryonic stem cells are murine or human embryonic stem cells. Accordingly, Applicant submits that this rejection has been rendered moot. Applicant respectfully requests that this rejection be reconsidered and withdrawn.

C. Rejection regarding "genetically modified human ES cells"

Furthermore, the Office Action states that since the claims continue to encompass the use of genetically modified human ES cells, the claims are rejected for lack of an enabling disclosure. (Office Action, page 7)

Applicant does not necessarily agree with the determination that claims to genetically-modified embryonic stem cells are not enabled. Furthermore, Applicant does not agree with the legal analysis that the broader independent claims are not enabled because they continue to encompass the use of genetically modified human embryonic stem cells.

However, in order to expedite prosecution, Applicant has amended the independent claims to recite that the embryonic stem cells are "not human genetically modified embryonic stem cells." The specification refers to "genetic modification of ES cells" at page 3, lines 3-4 and states in original claim 13 that the "cells were genetically modified." M.P.E.P. § 2173.05(i) states that the "current view of the courts is that there is nothing inherently ambiguous or uncertain about a negative limitation." Furthermore, this section states that if "alternative elements are positively recited in the specification, they may be explicitly excluded in the claims. See *In re Johnson* . . . ." (Citations omitted) Accordingly, Applicant submits that this new claim limitation is adequately supported by the specification as filed and does not constitute new matter. Applicant respectfully requests that this rejection be reconsidered and withdrawn.

Applicant respectfully submits that all of the facets of this enablement rejection of claims 2, 3, 6, 8-12, 15, 46-48, 50, 76-83, 85-94, and 96-104 under 35 U.S.C. § 112, first paragraph, have been rendered moot, and that new claims 106-136 are enabled. Accordingly, Applicant requests that this rejection be reconsidered and withdrawn.

III. New Claims Corresponding to Claims 46, 86, 97, and 99 as Previously Filed Do Comply with the Enablement Requirement

Claims 46, 86, 97, and 99 stand rejected under 35 U.S.C. § 112, first paragraph for allegedly failing to comply with the enablement requirement.

Applicant notes that these claims have been cancelled herein without prejudice or disclaimer of the subject matter contained therein. These claims have generally been rewritten as claims 115, 125, and 134.

The Office Action states that with regard to the rejection of claim 46, 86, 97, and 99 for lack of an enabling disclosure for producing a therapeutic effect upon transplantation of the claimed cell compositions, there is nothing in Example 4 (pointed to by Applicant) that points to a therapeutic outcome, and Applicant provides no support for the assertion that Example 4 provides a therapeutic use. Applicant respectfully disagrees.

M.P.E.P. 2164.01(c) states that

> [W]hen a compound or composition claim is not limited by a recited use, any enabled use that would reasonably correlate with the entire scope of that claim is sufficient to preclude a rejection for nonenablement based on how to use. . . . In other words, if any use is enabled when multiple uses are disclosed, the application is enabling for the claimed invention. (Emphasis added)

The Office Action admits that an enabling disclosure for a pharmaceutical use of claims 48, 86, and 99 has been provided based on Example 5.2. Thus, for at least these claims, at least one use has already been shown.

Applicant further provides an additional therapeutic use, *i.e.* the transplantation of oligodendroglial /astrocytic precursors into myelin-deficient rats (for the purposes of myelin regeneration necessary for proper neural conduction), which is fully described in Example 4 (beginning on page 26, line 35) and throughout the specification. Applicant submits that the model used in Example 4 (myelin deficient rats) is a model for Pelizaeus-Merzbacher disease (PMD), as would be well known to one of skill in the art. For example, Lazzarini, Myelin Biology and Disorders Vol. 2, Chapter 47; Models of Pelizaeus-Merzbacher Disease, Elsevier Academic Press (2004) p. 1130 (attached hereto as Appendix A) indicates that the myelin-deficient, *md rat*, had been identified and provides a model for the connatal form of Pelizeaus-Merzbacher Disease associated with a lack of PLP. Furthermore, Koeppen *et al.*, (1987) *Ann. Neurol.* 21:159-70 (attached hereto as Appendix B), indicates that "Pelizaeus-Merzbacher disease in humans shares many neuropathological and biochemical features with X-linked mutations in animals, e.g., the jimpy mouse and myelin-deficient rat." (Abstract) Additionally, Applicant submits that Example 4 shows a therapeutic use, since it shows that donor mouse cells were detected in the rat brain following transplantation (which fact was admitted in the Office Action at page 7). In PMD, patients have a problem with the myelin-coating gene PLP, and as a result, the patients have a problem with forming myelin. Thus, the presence of donor-derived myelin formation (see page 28, lines 16-18) would be considered therapeutic. There are many instances where there is no measurable therapeutic effect other than to restore anatomical structure. Such examples (e.g. keratinocyte transplants for skin reconstruction or blood vessel transplants) would be well known to one of skill in the art. Thus, one of skill in the art would be aware that the presence of donor mouse cells detected in the rat brain following embryonic transplantation is a therapeutic effect.

Accordingly, Applicant respectfully submits that this rejection has been overcome. Applicant respectfully requests that this rejection be reconsidered and withdrawn.

IV. New Claims Corresponding to Claims 2, 3, 6, 8-12, 15, 46-48, 50, 76-83, 85-94, and 96-104 as Previously Filed Are Not Anticipated by U.S. Patent No. 5,980,885

Claims 2, 3, 6, 8-12, 15, 46-48, 50, 76-83, 85-94, and 96-104 were rejected under 35 U.S.C. § 102(e) as allegedly being anticipated by U.S. Patent No. 5,980,885 (Weiss *et al.*). These claims have been cancelled herein without prejudice or disclaimer of the subject matter contained therein. These claims generally correspond to new claims 106-136.

The Office Action states that the instant claims are product-by-process claims, which are not limited to the manipulations of the recited steps, only the structure implied by the steps; thus, the claims read on neural stem cells disclosed in the prior art. (Office Action, pages 8-9) The Office Action further states that in the absence of evidence to the contrary, the neural stem cell compositions disclosed by Weiss et al. are indistinct from the cell compositions instantly claimed. (Office Action, page 9) Applicant disagrees.

M.P.E.P. 2113 states that

> The structure implied by the process steps should be considered when assessing the patentability of product-by-process claims over the prior art, especially where the product can only be defined by the process steps by which the product is made, or where the manufacturing process steps would be expected to impart distinctive structural characteristics to the final product. (emphasis added)

Applicant respectfully submits that the neural stem cell compositions disclosed by Weiss et al. are distinct from the instantly claimed cell compositions obtained as recited in the claims.

As discussed in the Second Declaration of Dr. Oliver Bruestle Under 37 C.F.R. § 1.132, neural precursor cells derived from neural tissue (embryonic, juvenile or adult) according to the Weiss method maintain their regional identity. Thus, neural precursor cells derived from a specific brain region are restricted. (paragraph 8) Thus, at least some neural stem cells or neural precursor cells obtained from neural tissue will express

region-specific genes, even when removed from the specific region of the brain and cultured *in vitro*. Furthermore, the neural precursor cells derived from a specific brain region will form the types of neurons found in this region. (paragraph 14) In contrast, the cell compositions of the currently pending claims are derived from embryonic stem cells, not neural tissue. Accordingly, these cells will not exhibit the region-specific expression profile that would be seen in the cells of Weiss *et al.*. They are not restricted with regard to the region-specific markers that they can express and the types of neural cells that they can become. Accordingly, one of skill in the art would know that the claimed cell compositions are different from those described in Weiss *et al.* (paragraph 15) Neural precursor cells derived from embryonic stem cells of the present invention have the potential to generate all cell types of the nervous system not confined by any particular region. (paragraph 8) Thus, in this application, the preparation process, which involves an initial step of culturing embryonic stem cells, imparts a distinctive structural characteristic to the final product.

Thus, the final product cell compositions of the claimed embodiments of the invention are distinct from those of Weiss *et al.* and are not anticipated by the Weiss *et al.* patent.

Accordingly, Applicant submits that this rejection has been overcome. Applicant respectfully requests that this rejection be reconsidered and withdrawn.

CONCLUSIONS

In view of the arguments set forth above, Applicant respectfully submits that the rejections contained in the Office Action mailed on December 2, 2005, have been overcome, and that the claims are in condition for allowance. It is believed that all of the pending claims have been addressed. However, the absence of a reply to a specific rejection, issue, or comment does not signify agreement with, or concession of, that rejection, issue, or comment. In addition, because the arguments made above may not be exhaustive, there may be reasons for patentability of any or all pending claims (or other claims) that have not been expressed. Finally, nothing in this paper should be construed as an intent to concede any issue with regard to any claim, unless specifically stated in this paper, and the amendment of any claim does not necessarily signify concession of unpatentability of the claim prior to its amendment.

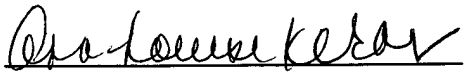
Applicant encloses herewith a Petition for a Two Month Extension of Time up to May 2, 2006 for responding to the Office Action dated December 2, 2005. Please charge our Deposit Account No. 08-0219 the $225.00 fee (small entity) for this extension of time.

Please also charge our Deposit Account No. 08-0219 the $395.00 fee (small entity) for filing a Request for Continued Examination.

No other fees are believed to be due in connection with this response. However, please charge any underpayments or credit any overpayments to Deposit Account No. 08-0219.

If the Examiner has any questions or amendments that she would like to discuss with the Applicant, she is encouraged to call the undersigned at the number indicated below.

Respectfully submitted,



Ann-Louise Kerner, Ph.D.
Reg. No. 33,523

Date: April 28, 2006

WILMER CUTLER PICKERING
HALE AND DORR LLP
60 State Street
Boston, MA 02109
Tel: (617) 526-6000
Fax: (617) 526-5000

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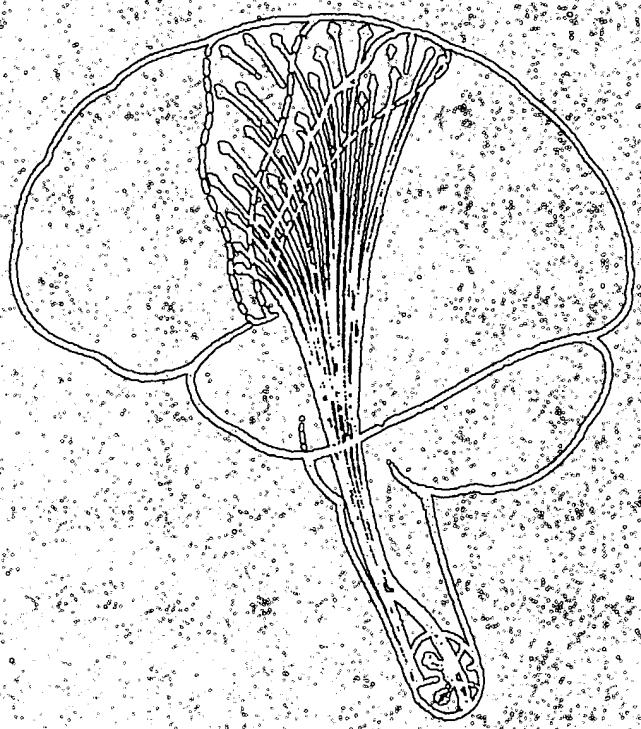

Section Editors:

Robert A. Lazzarini • John W. Griffin • Hans Lassma
Klaus-Armin Nave • Robert H. Miller • Bruce D. Trap

Elsevier Academic Press
525 B Street, Suite 1900, San Diego, California 92101-4495, USA
84 Theobald's Road, London WC1X 8RR, UK

This book is printed on acid-free paper. ♾

Library of Congress Cataloging-in-Publication Data

Lazzarini, Robert A.
Myelin biology and disorders / Robert A. Lazzarini
p. cm.
Includes index.
ISBN 0-12-439510-4 (alk. paper)
1. Myelin sheath. 2. Myelin sheath–Pathophysiology. 3. Myelin sheath–Diseases–Animal models. 4. Demyelination. I. Title

QP752.M9L39 2003
612.8'1–dc22

2003062808

British Library Cataloguing in Publication Data
A catalogue record for this book is available from the British Library

International Standard Book Number: 0-12-439510-4 (set)
International Standard Book Number: 0-12-439511-2 (vol. 1)
International Standard Book Number: 0-12-439512-0 (vol. 2)

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Printed in China
03 04 05 06 07 08 9 7 6 5 4 3 2 1

The *Myelin-Deficient* Rat

The myelin-deficient *md rat* has been identified by Csiza (Csiza and Lahunta, 1979; Dentinger *et al.* 1982) and also provides a model for the connatal form of PMD associated with a lack of PLP (Koeppen *et al.*, 1987). Boison and Stoffel (1989) identified the primary defect in the rat *Plp* gene, associated with the nonconservative substitution T74P and predicting a break in the second transmembrane helix of PLP. The complete lack of mutant PLP from compact myelin results in a characteristic decrease of myelin periodicity—that is, closer apposition of myelin membranes (Duncan *et al.*, 1987)—later confirmed in *jimpy* mice (Duncan *et al.*, 1995; Schneider *et al.*, 1995), as well as subtle axonal abnormalities (Barron *et al.*, 1987; Dentinger *et al.*, 1985). Clinically, *md rats* are similar to the *Plp* mutant mice, with tremors, tonic seizures, and premature death. Recently, a longer-lived mutant substrain has been isolated in which affected males survive for about 80 days (Duncan *et al.*, 1995), suggesting the importance of yet unknown modifier genes. As larger animals, *md rats* are well suited for cell transplantation experiments (Bruestle *et al.*, 1999), for electrophysiological studies on the consequences of myelin-deficiency (Utzschneider *et al.*, 1992; Young *et al.*, 1989), and for research on the underlying cause of premature death (Miller *et al.*, 2003).

The *Rumpshaker* Mouse

The *rumpshaker* ($Plp^{jp\text{-}rsh}$) mutation predicts the substitution Ile-186-Thr in PLP and DM20, and is associated with a relatively mild phenotype (Schneider *et al.*, 1992). The preservation of oligodendrocytes with considerably reduced cell death, when compared to *jimpy*, results in substantially improved myelination (Fig. 47.3) (Griffiths *et al.*, 1990). The optic nerve contains both amyelinated and normally myelinated axons, whereas spinal cord axons are only thinly myelinated with little growth in thickness during development (Fanarraga *et al.*, 1992). Different from *jimpy* and *jimpy-msd* mice, *rumpshaker* mutant myelin also incorporates significant amounts of proteolipids, with the DM20 isoform predominating (Fanarraga *et al.*, 1992; Karthigasan *et al.*, 1996). However, the altered ratio of PLP/DM20 is not responsible by itself for a dysmyelinated phenotype (Uschkureit *et al.*, 2001). Karthigasan *et al.* (1996) reported that the biochemical abnormalities in *rumpshaker* myelin correlate with a wider periodicity and less stable packing of the layers. *Rumpshaker* mice also demonstrated that apoptosis in other *Plp* mutants is not caused by dysmyelination per se (Schneider *et al.*, 1992), but as discussed later, rather by the toxicity of mutant PLP and DM20 isoforms when retained in the endoplasmic reticulum of oligodendrocytes (Gow and Lazzarini, 1996; Gow *et al.*, 1998).

Rumpshaker mice are long lived and reproduce well, although showing ataxia and mild tremor, but little seizure activity. Interestingly, the severity of their phenotype is markedly influenced by the background strain—that is, unknown modifier genes (Al-Saktawi *et al.*, 2003) (Fig. 47.4). *Rumpshaker* mice are a good animal model for SPG-2 in humans, in fact the same point mutation (I186T) was identified later in the index family of this human disease (Kobayashi *et al.*, 1994; Naidu *et al.*, 1997).

PLP/DM20-DEPENDENT DISEASE MECHANISMS IN NATURAL AND ENGINEERED RODENT MODELS OF PMD AND SPG-2

Pelizaeus-Merzbacher disease and SPG-2 have been associated with (and are now genetically defined by) mutations of the human *PLP* locus, specifically (1) various point mutations in the coding region of the *PLP* gene and consequently the expression of mutant proteolipids, (2) with the loss of PLP function in null alleles or equivalent mutations, and (3) with the overexpression of PLP in patients that carry a gene duplication. For an updated listing of specific mutations in the *PLP* gene, see www.med.wayne.edu/Neurology/plp.html.

At the molecular level, it appears that PMD/SPG-2 is associated with three distinct pathomechanisms that act overlappingly. They have been revealed by dissecting the disease process at the cellular level in cultured cells and in corresponding mouse mutants.

Defective Biosynthesis of Proteolipid Protein in Pelizaeus-Merzbacher Disease

Arnulf H. Koeppen, MD,*† Nicholas A. Ronca, MS,* Edward A. Greenfield, PhD,* and Mary B. Hans, MSW*†

The brain of an 18-year-old patient with Pelizaeus-Merzbacher disease was examined by standard neuropathological and biochemical methods and by immunocytochemical and immunochemical techniques. Analysis revealed a lack of myelin-specific lipids, but showed a residual immunoreactivity for myelin basic protein, myelin-associated glycoprotein, and 2',3'-cyclic nucleotide-3'-phosphodiesterase. Examination by immunocytochemistry and enzyme-linked immunosorbent assay showed an absence of proteolipid apoprotein (lipophilin). The peripheral nervous system was normal. Pelizaeus-Merzbacher disease in humans shares many neuropathological and biochemical features with X-linked mutations in animals, e.g., the jimpy mouse and myelin-deficient rat. The specificity of this protein deficiency in Pelizaeus-Merzbacher disease gains additional support from the recent mapping of the lipophilin gene to the human X chromosome.

Koeppen AH, Ronca NA, Greenfield EA, Hans MB: Defective biosynthesis of proteolipid protein in Pelizaeus-Merzbacher disease. Ann Neurol 21:159–170, 1987

Pelizaeus-Merzbacher disease (PM) (McKusick No. 31,160 [32]) is a rare X-linked disorder of boys and young adult men characterized by a deficiency of myelin in the central nervous system (CNS). Based on clinical features and the degree of myelin deficiency, different types have been recognized [45]. The peripheral nervous system (PNS) is intact. Several mutant animals with an X-linked lack of CNS myelin exist, among them the jimpy mouse [47], the myelin-deficient rat [7], the shaking pup [16], and the Landrace pig with congenital tremor [17].

Neuropathological and biochemical studies of PM have been quite numerous, but the genetically determined biochemical lesion has remained elusive. The assembly of myelin in the PNS of patients with PM is remarkably intact, so the answer may be sought in the known chemical differences between CNS and PNS myelin. Proteolipid and its apoprotein proteolipid protein (PLP) (or lipophilin) come to mind because the PNS is devoid of immunoreactive PLP. This report is based on the morphological, chemical, and immunochemical analyses of brain tissue from a patient with PM. The evidence points toward a genetic defect of PLP biosynthesis that prevents proper differentiation and survival of oligodendrocytes. The biosynthesis of three other myelin proteins, myelin basic protein (MBP), myelin-associated glycoprotein (MAG), and 2',3'-cyclic nucleotide-3'-phosphodiesterase (CNP) (E.C. 3.1.4.37), is at least partially preserved.

As PM is an X-linked disorder, the gene for PLP biosynthesis may be localized to the human X chromosome. Complementary deoxyribonucleic acid (cDNA) probes for bovine and rat PLP were developed quite recently [8, 9, 34, 36] and found to hybridize exclusively with the X chromosome (Xq13-Xq22 in humans) [58].

Family Study and Case Reports

The pedigree of the family with PM is illustrated in Figure 1. X-linkage and the carrier status of some female members of the family are apparent. The sex of unborn children IV,4 and IV,6 was determined after amniocentesis, and the daughters of these pregnancies have shown no evidence of neurological disease. The diagnosis of PM in II,4 and II,5 is somewhat tentative but was strongly supported by descriptions obtained from their mother (I,1), their living half-sister (II,3), and the two death certificates, with respective diagnoses of "flaccid paralysis since birth" (II,4) and "torticollis, scoliosis, talipes, Erb's palsy" (II,5). Case notes were obtained from the family physician of II,5. He reported that the child was normal at birth but developed "early ossification of the skull bones at 3 months and progressive spastic paraparesis."

Patient III,7 (Propositus)

The mother of Patient III,7 reported a normal pregnancy and delivery at term. His birth weight was 2,920 gm (6 lb, 7 oz) and his head circumference was 41 cm. He failed to thrive and was first admitted to the hospital at the age of 2

From the *Neurology Service, Veterans Administration Medical Center, and the †Department of Neurology, Albany Medical College, Albany, NY.

Received Apr 14, 1986, and in revised form June 19. Accepted for publication June 19, 1986.

Address reprint requests to Dr Koeppen, Neurology Service (127), VA Medical Center, Albany, NY 12208.

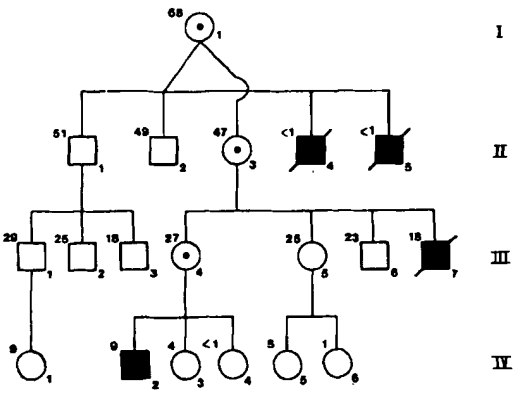


Fig 1. Pedigree of the family with Pelizaeus-Merzbacher disease. Patients with clinical features of the disease are shown by solid symbols. Obligatory, clinically normal female carriers are shown by a dot in the center of the circles. The females III,5; IV,3; IV,4; IV,5; and IV,6 may or may not be carriers. The assignment of family members II,4 and II,5 as "affected" is based on the report by their living mother (I,1) and half-sister (II,3), the family physician's case notes, and death certificates. Neurological examinations were performed in Patients III,7 (now deceased) and IV,2. A postmortem examination was performed on Patient III,7.

months because of a generalized seizure. Examination revealed closure of the anterior fontanelle, hypotonia, and total areflexia. He was treated with phenobarbital. Many additional hospitalizations followed, and phenytoin was added to his anticonvulsant regimen. Werdnig-Hoffmann disease was suspected because his extremities remained thin and he had fasciculations of the tongue. Electromyography of his extremity muscles done at 4 months of age revealed denervation. However, nerve and muscle biopsy findings were normal. The diagnosis of Werdnig-Hoffmann disease was rejected after vision failure and mental retardation became evident. His head and body failed to grow, and at 2 years his head circumference was 46 cm and his weight was 6.8 kg (15 lb). A biopsy of the rectal mucosa did not support the diagnosis of lipidosis. Lumbar puncture yielded normal cerebrospinal fluid. Seizures were incompletely controlled, and he required a feeding gastrostomy. The diagnosis of PM was first suggested when his nephew (IV,2) showed symptoms and signs of the same disease. A computed tomographic (CT) scan done when the propositus was 16 years old showed smallness of the head, widened subarachnoid spaces over the cerebral hemispheres and the brainstem, internal hydrocephalus, and no distinction between gray and white matter. He was examined by one of us (A.H.K.) at age 17. He did not respond to voice or tactile stimuli. His head circumference was 50 cm, and he weighed 15.4 kg (34 lb). Occasional myoclonic jerks occurred. His pupils reacted to light but he had severe optic atrophy. There was no cherry-red spot. His spontaneous eye movements were slow and dysjunctive, but brisk with passive motion of the head. Corneal reflexes were present. He vocalized only occasionally. He was quadriplegic and areflexic, and his extremities were extremely thin. Massive gingival hyperplasia and some hirsutism had resulted from the phenytoin therapy. Skin biopsy and blood samples were collected for fibroblast and lymphoblast cultures. His karyotype was normal. He died at age 18 from bronchopneumonia, and an autopsy was performed within 3 hours.

Patient IV,2

At age 7 years the nephew of the propositus was examined by A.H.K. His mother reported poor and infrequent intrauterine movements compared to her subsequent female pregnancy (IV,3). Patient IV,2 was born at term, weighing 3,713 gm (8 lb, 3 oz). Because of his peculiar cry, a diagnosis of cri du chat syndrome was first considered but was later changed to Werdnig-Hoffmann disease when fasciculations of his deltoid muscles developed. He remained hypotonic but sucked well and rolled over. He never sat without support. Blindness was first suspected at 10 to 12 months, and seizures occurred at 17 months, which were treated with carbamazepine but recurred frequently. Biopsies of skin, muscle, nerve, and rectal mucosa were inconclusive. When examined at the age of 7, he had a head circumference of 47 cm, weighed 7.7 kg (17 lb), and was 91 cm long. All measurements were below the second percentile. He had scoliosis of the thoracic spine. He did not respond to visual stimuli but appeared to recognize his mother's familiar voice. Though his head control was poor, he had opisthotonos and tonic neck reflexes. His pupils reacted well to light, and the optokinetic tape elicited some jerk nystagmus. The optic discs were white. Cherry-red spots were absent. He had little spontaneous movement of the extremities and flail hands and feet. He held his arms in flexion, but his legs were spastic in extension. All muscle stretch reflexes and toe responses were absent. In the 2 years since this examination, seizure control and feeding have become more difficult.

Morphological Methods

Both frontal lobes of Patient III,7 were frozen for biochemical analysis, and pieces of cortex and subcortical white matter were fixed for 24 hours in ice-cold 4% paraformaldehyde in phosphate buffer (pH, 7.2). The fixed tissue was then transferred into a solution of 15% sucrose in phosphate-buffered saline (PBS) containing 15mM sodium azide. These specimens were kept at 4°C until sectioned for immunocytochemical analysis of MBP, MAG, CNP, PLP, microtubule-associated protein 2 (MAP 2), neuron-specific enolase (NSE) (E.C. 4.2.1.11,γ,γ), and nonneuronal enolase (NNE) (E.C. 4.2.1.11,α,α). The remaining brain tissue, one eye, and the spinal cord were fixed in neutral formaldehyde solution (4%). After 10 days, samples were embedded in paraffin and sectioned for routine stains, including hematoxylin-eosin; cresyl violet; Luxol fast blue–periodic acid–Schiff (LFB-PAS) (myelin); and Bodian silver protein (axons). Paraffin sections were also selected for the immunocytochemical visualization of glial fibrillary acidic protein (GFAP) and the S-100 protein.

Samples of formaldehyde-fixed brain and spinal cord were examined by established methods of electron microscopy.

Sucrose-impregnated cortex and subcortical tissue of Patient III,7 and similarly processed normal control specimens were sectioned at 40 μm with a Lancer 1,000 vibrating microtome (vibratome). Preliminary studies showed that anti-

sera to MBP, MAG, CNP, PLP, and a PLP-related synthetic peptide [11] did not readily reach antigenic sites in the myelin sheaths of tissue sections from normal adult brain unless they were "unmasked" by physical or chemical means. This phenomenon has been most extensively studied with MBP [19, 33, 37, 51, 52], but similar difficulties were found with antisera to MAP, CNP, and PLP. After trial and error, the following procedure was adopted to reveal the antigenic sites: Floating vibratome sections were washed in ice-cold distilled water and then incubated overnight at 4°C in 95% ethanol (MBP, MAG, PLP) or for 1 hour at room temperature (CNP). For the visualization of MAP 2, NSE, and NNE, the ethanol incubation was omitted. Ethanol was removed by washing in PBS. The sections were then successively incubated in 0.1 M neutralized periodate (15 min), sodium borohydride (5 mg/ml; 10 min), and 5% dimethyl sulfoxide in PBS (30 min). Each of the above steps was separated by washing with PBS (3 × 10 min each). After dimethyl sulfoxide was removed by PBS, the sections were incubated for 1 hour at room temperature with a "suppressor serum" to quench nonspecific background staining. It contained 10% normal goat serum, 4% bovine serum albumin, and 0.1% Triton X-100 (a nonionic detergent). Suppressor serum was drained off without washing, and the sections were transferred into a dilute solution of antiserum. Dilutions were 1:1,000 for antisera to MBP, MAG, CNP, PLP-related peptide, MAP 2, NSE, and NNE, and 1:100 for PLP (apoprotein). The solutions also contained 1% normal goat serum and 0.1% Triton X-100. Incubations were at 4°C overnight. Unreacted antibody was then removed by PBS washes, and the sections were immersed in diluted biotinylated antirabbit IgG (available in kit form from Vector Labs, Burlingame, CA). An avidin-biotin-peroxidase complex (ABC) was then fixed to the biotinylated sites, as described in the literature [22] and by the supplier of the ABC kits. The chromogenic substrates were diaminobenzidine tetrahydrochloride and 0.01% hydrogen peroxide. The reaction product was enhanced by brief exposure to 1% osmium tetroxide in phosphate buffer, the sections were washed in water, mounted onto chromalum-gelatin–coated glass slides, dehydrated, and coverslipped.

Paraffin sections for the immunocytochemical analysis of GFAP and the S-100 protein were fixed to slides with a thin layer of glue (Bond Fast white glue; LePage, Bramalea, Ontario, Canada). The sections were deparaffinized with toluene and rehydrated with a series of diminishing concentrations of ethanol and water. Quenching of endogenous peroxidase activity was achieved by incubation in methanol containing 3% hydrogen peroxide. Exposure to periodate, sodium borohydride, and dimethyl sulfoxide was omitted. The sections were incubated for 20 minutes in a neutralized solution of trypsin (1 mg/ml) and calcium chloride (1 mg/ml). The application of suppressor serum, primary immune serum, biotinylated antibody, and ABC, as well as intervening washing with PBS and color development, were as described for the vibratome sections.

Biochemical and Immunochemical Methods

Lipid Extraction and Analysis

The term *white matter* is used here to refer to both normal and PM-affected brains, even though the subcortical tissue from the patient with PM was devoid of normal myelin. Cortical gray and white matter were separated under a magnifying lens.

Lipids were extracted and purified as described by Folch and colleagues [14], and PLP was removed as reported by Webster and Folch [57]. Polar lipids were separated by two-dimensional thin-layer chromatography [40]. Adequate separation of cerebrosides and sulfatides was achieved, but no effort was made to distinguish between the hydroxy and nonhydroxy fractions of these lipids. Neutral lipids (cholesteryl esters, cholesterol, triacylglycerols, diacylglycerols, and monoacylglycerols) were separated on silica gel layers (250-μm thickness) in a solvent system of petroleum ether–diethylether–acetic acid (45:5:0.5, by vol).

Phospholipid phosphorus was assayed according to Chen and associates [6], and lipid galactose as described by Hess and Lewin [20]. The colorimetric reaction of Searcy and Bergqvist [43] was adapted for the extracted and purified cholesterol and cholesteryl esters. Acylglycerol concentrations were estimated by the method of Carlson [5], after modification [27].

Total and Myelin Proteins

Total proteins were assayed according to Sedmark and Grossberg [44], and purified proteolipid protein by the method of Lees and Paxman [30]. Sample preparation for sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and enzyme-linked immunosorbent assay (ELISA) differed substantially for the myelin proteins of interest and is summarized as follows. Aliquots of normal and affected white matter were weighed, homogenized, and then lyophilized. The dry material was partially delipidated by a five-time extraction with a mixture of diethylether and ethanol (3:2, by vol; 1 ml/mg dry weight). The residue was dried under a stream of nitrogen and dissolved in sample buffer before SDS-PAGE (11% acrylamide) [29]. This procedure yielded satisfactory results for MBP, MAG, CNP, and GFAP. For SDS-PAGE of PLP, the apoprotein was obtained as described by Bizzozero and co-workers [3].

After elution of the void volume from the Sephadex LH 60 column, one-half volume of chloroform was added to the pooled fractions. The mixture was concentrated at 30°C in a rotary evaporator while water suction was applied. Small volumes of chloroform were added to keep the solution clear. The apoprotein was then precipitated over a period of 4 hours with 5 volumes of acetone at −20°C and collected at 4°C by centrifugation. The supernatant was removed by aspiration, and the pellet was partially dried with a stream of nitrogen. A sufficient volume of a mixture of chloroform–methanol (2:1, by volume) was added to dissolve the pellet. Seven volumes of benzene were added to this mixture [2]. The solution was then lyophilized. The undesirable aggregation of PLP apoprotein did not occur. Aliquots of the dry protein were taken up in sample buffer [29] from which 2-mercaptoethanol was omitted [35]. The solution became clear after about 1 hour at room temperature and was then ready for SDS-PAGE. Electrophoresis of the solubilized proteins and standards of known molecular weight was performed on 11% polyacrylamide gels. A portion of the gel was stained with Coomassie Brilliant Blue G 250, de-stained, and photographed. Proteins of the remaining gel were trans-

ferred to nitrocellulose paper (pore size, 0.45 μm) [54] and revealed by immunochemical methods ("immunoblotting") as follows. Nitrocellulose sheets were cut into strips for the visualization of MBP, MAG, CNP, GFAP, and PLP. The paper was saturated for 1 hour at 40°C with 10% normal goat serum, which also contained 4% bovine serum albumin. After draining this blocking serum, the strips were incubated at 4°C overnight with the respective rabbit polyclonal antisera, diluted as for the tissue sections. The washing solutions contained PBS, 1% normal goat serum, and 0.1% Triton X-100. The further sequence included biotinylated antirabbit immunoglobulin G and ABC, as described for the vibratome sections. Color was developed from diaminobenzidine tetrahydrochloride and hydrogen peroxide, but imidazole enhancement [53] replaced the osmium tetroxide step. MAG content in the brain of the PM patient was also estimated by Drs Johanna Moller and Richard H. Quarles, who applied the highly sensitive method detailed in [61].

For ELISA, partial purification of MBP and CNP followed procedures described in the literature (MBP [12]; CNP [10, 24, 49]). The purification was advanced to the point of first chromatography. Water-soluble PLP apoprotein was prepared as described by Macklin and Lees [31] and used as a standard for PLP-ELISA. For ELISA of MAG, samples of white matter were homogenized in water, lyophilized, delipidated with ether-ethanol [35] as for SDS-PAGE, and dissolved in a solution of 1% SDS in water. After heating the mixture at 100°C for 5 minutes [25], the insoluble residue was removed by centrifugation and the clear supernatant was exhaustively dialyzed against water to remove free SDS. Samples of purified MBP were available for quantitative ELISA, but comparative analysis was done only for MAG and CNP. In each case, samples of normal affected white matter were processed in an identical manner. ELISA procedures were guided by the manual of Voller and colleagues [55]. The coating procedure of polystyrene plates varied with the antigenic protein. Dilute MBP was applied in 0.01 M sodium phosphate buffer (pH, 6.0). After 4 hours, the wells were washed with PBS, then filled with a solution of bovine serum albumin (1%) and histones (0.1%) in PBS, and allowed to saturate the surface of the wells with irrelevant proteins (24 hours at 4°C). All washing steps were done with PBS containing 1% bovine serum albumin, 0.1% histones, and 0.05% Tween 80 (polysorbate 80). Anti-MBP was diluted 1:1,000 in this buffer and allowed to react with the fixed antigen at 4°C overnight. After washing, horseradish peroxidase–labeled goat antirabbit gamma globulin (diluted 1:200 in the washing buffer) was added. After incubation for 4 hours at room temperature and the intervening washing steps, color was developed with *o*-phenylenediamine and hydrogen peroxide, as described [55]. ELISA of MAG, CNP, and PLP was done in a similar way but the coating was done in carbonate–bicarbonate buffer (pH, 9.6) [55]. Nonspecific background color was suppressed by incubation with 1% bovine serum albumin in washing buffer.

The enzymatic activity of CNP was assayed in acetone powder extracts, as described by Hugli and associates [24].

Sources of Antisera for Immunocytochemistry and ELISA

Antisera to MBP, MAG, CNP, PLP, PLP-peptide, MAP 2, and NNE were generously provided by other scientists (see Acknowledgment). Antisera to GFAP, NSE, and S-100 were obtained from DAKO Labs, Santa Barbara, CA. Horseradish peroxidase–labeled goat antirabbit gamma globulin was purchased from Antibodies, Inc, Davis, CA.

Results

Neuropathological Observations

The unfixed brain of Patient III,7 weighed 830 gm (expected: 1,400 gm), was greatly reduced in size, and atrophic. The basis pontis was thinned, and the cerebellum was small and firm. The cisterna magna and vallecula were enlarged due to shrinkage of the vermis and cerebellar hemispheres. The pyramidal tracts were hypoplastic. Optic nerves, chiasm, and tracts lacked their usual white color and appeared gelatinous, whereas the other cranial nerves were normal (Fig 2). Coronal slices of the brain showed internal hydrocephalus and poor demarcation between gray and white matter (Fig 3). The corpus callosum was reduced to a thin ribbon, and the color of the pallidum did not differ from that of the putamen (Fig 3). The fornices were thin, gray, and gelatinous. The thickness of the cerebral cortex was not abnormal, and the basal ganglia and diencephalon were also of approximately normal dimensions. Transverse sections of the brainstem revealed the atrophic basis pontis, dilatation of the fourth ventricle, and atrophy and sclerosis of the cerebellum. The spinal cord was reduced in size but had normal anterior and posterior roots. On transverse sections, the long tracts were gray, and the demarcation between gray and white matter was not sharp.

On routine histological staining, the cortex was of normal thickness and showed regular lamination. However, satellite oligodendrocytes and myelin sheaths were absent. The centrum semiovale contained no characteristic oligodendrocytes and was devoid of myelin. Astrocytes predominated but many cells had cyto-

Fig 2. Brainstem of the patient with Pelizaeus-Merzbacher disease. The normally myelinated cranial nerves 3, 5, and 6 stand out in contrast to the gray and gelatinous basis pontis.

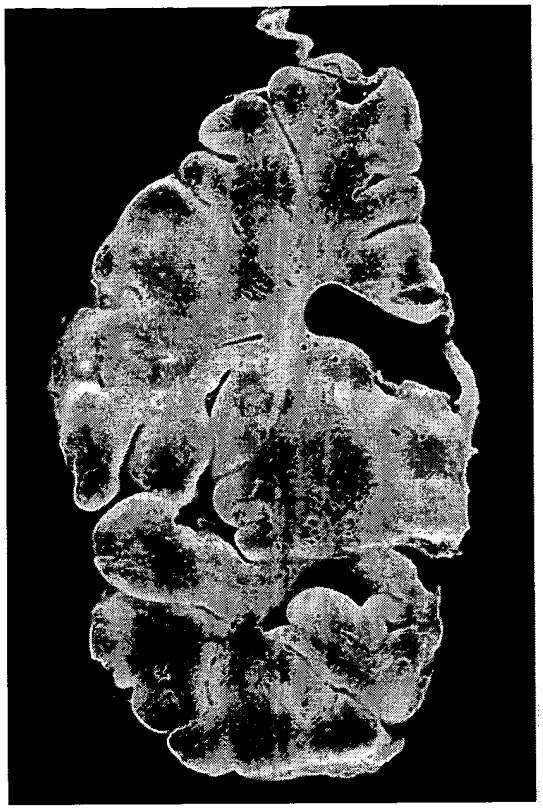


Fig 3. Coronal slice of the left cerebral hemisphere of the patient with Pelizaeus-Merzbacher disease. Cortex and white matter are not sharply demarcated. The corpus callosum is very thin. The globus pallidus lacks its normal lighter color and appears to blend with the adjacent putamen. The internal capsule is thin and indistinct.

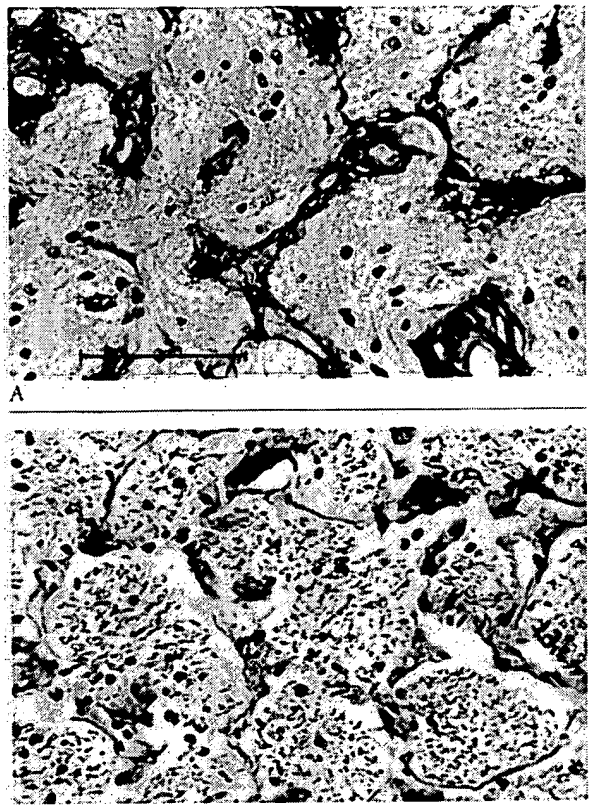


Fig 4. Optic nerve in Pelizaeus-Merzbacher disease. The Luxol fast blue–periodic acid–Schiff stain (A) *does not reveal any myelin sheaths, whereas the Bodian technique* (B) *demonstrates abundant axons. (Bar = 100 μm.)*

plasmic inclusions that stained well with cresyl violet, suggesting a neuronal origin (see following discussion of MAP 2 immunocytochemistry). The basal ganglia and diencephalon had normal nerve cell densities but were devoid of myelin and juxtaneuronal oligodendrocytes. At brainstem levels, nerve cells were also of normal abundance, but the fiber tracts lacked oligodendrocytes and myelin. Silver stains showed many axons in all areas that were devoid of LFB-PAS–stainable myelin. The cerebellum showed a total loss of its cortical neurons. The folia and central portions contained no myelin but also revealed axonal loss. The cerebellar lesion was attributed to hypoxia resulting from the patient's severe seizures, and was thought to be superimposed on the myelin deficiency. However, the pyramidal band of the hippocampi revealed a normal nerve cell density. The optic nerves (Fig 4) contained no myelin sheaths but showed a near normal axonal density. Properly placed sections through the brainstem showed normal continuity of intraaxial and extraaxial cranial nerve axons. Myelin stains illustrated the sharp transition to normal cranial nerve myelin at a distance from the brainstem (Fig 5). Also, normal myelin was present in spinal roots (Fig 5, inset). The findings in the eye will be reported elsewhere. Despite the autolytic artifact, electron microscopy confirmed the total absence of myelin in the CNS. Myelination of the spinal roots was normal.

Immunocytochemical Observations

GFAP, S-100, AND NNE. Optic nerves, brain, and spinal cord white matter stained intensely using immunocytochemical methods for the astrocytic proteins GFAP, S-100, and NNE. Astrocytes in the cerebral cortex were not increased in number. These stains also revealed the junction of CNS and PNS at the exit (or entrance) points of cranial nerves and spinal roots.

MAP 2 AND NSE. Antisera to MAP 2 and NSE revealed a normal architecture of the cerebral cortex (Fig 6A). The cresyl violet–positive cells in the subcortical white matter of the PM patient gave a strong reaction product with anti–MAP 2 (Fig 6B) but not with anti-NSE. Their long dendrites showed random orientation but many were bipolar. These cells were considered

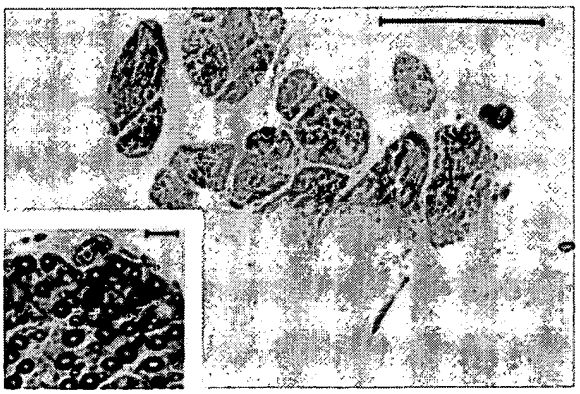


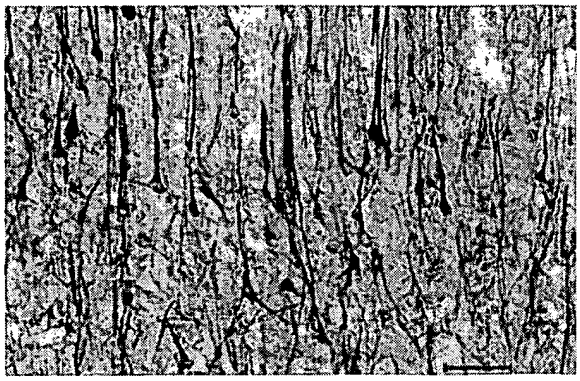
Fig 5. Exit of the abducens nerve from the central nervous system (CNS) in Pelizaeus-Merzbacher disease. Normal peripheral nervous system (PNS) myelin begins at a short distance from the brainstem. CNS myelin is absent. (Luxol fast blue–PAS stain; bar = 1 mm). Inset: Normal PNS myelin of a spinal nerve root. (Toluidine blue; bar = 25 μm.)

neurons with incomplete corticipetal migration and with immature conversion from NNE to NSE [41].

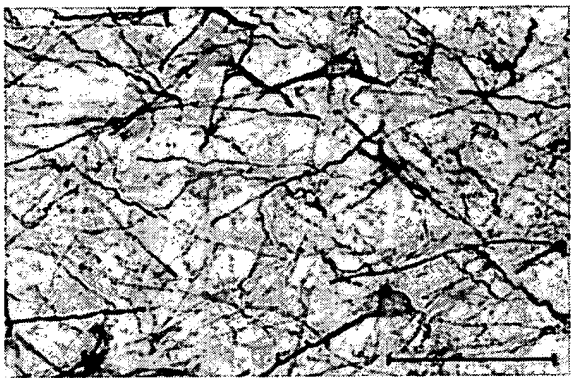
MBP, MAG, CNP, AND PLP. Immunocytochemical stains for MBP, MAG, CNP, and PLP are illustrated in Figure 7. Though devoid of lipid-stainable myelin, PM white matter contained islands of "myelin" when stained for MBP, MAG, or CNP. The orientation of these lipid-free myelin sheaths lacked a predominant direction and was haphazard. The cortex contained similar islands of myelin, though their number was less. The islands in PM were also seen when the preliminary incubation with ethanol was omitted, suggesting the unrestricted access of antisera to MBP, MAG, and CNP to their respective antigenic sites. When normal white matter was incubated without prior ethanol exposure, myelin sheaths were not revealed by antisera to MBP, MAG, CNP, or PLP. However, oligodendroglial cytoplasm reacted with anti-MBP, confirming earlier observations [52]. This cell-body reaction product disappeared after ethanol, but myelin reacted well. In the PM patient, antisera to MBP, MAG, and CNP revealed cell bodies and delicate processes that were attached to the fragmentary myelin sheaths (see Fig 7A–F). Though routine cell stains failed to show typical oligodendrocytes, the immunocytochemical methods illustrated cells that may be considered their equivalent. Anti-PLP (apoprotein) and anti-PLP-peptide showed no reaction product in the cortex or white matter of the PM patient (Fig 7H) but reacted well with normal white matter (Fig 7G).

Myelin Lipids and Proteins

Findings in gray and white matter from normal and PM brain are compared in Table 1. In comparing white matter, the deficiency of total lipid, galactolipids (cerebrosides and sulfatides), cholesterol, phosphatidylethanolamine, and sphingomyelin is especially severe. The lack of lipid and protein in white matter is also reflected in the high water content of the affected brain (88.8% versus 70.5% of normal white matter wet weight). Visual inspection of thin-layer plates after charring with sulfuric acid (not illustrated) revealed the total lack of cerebrosides and sulfatide spots.



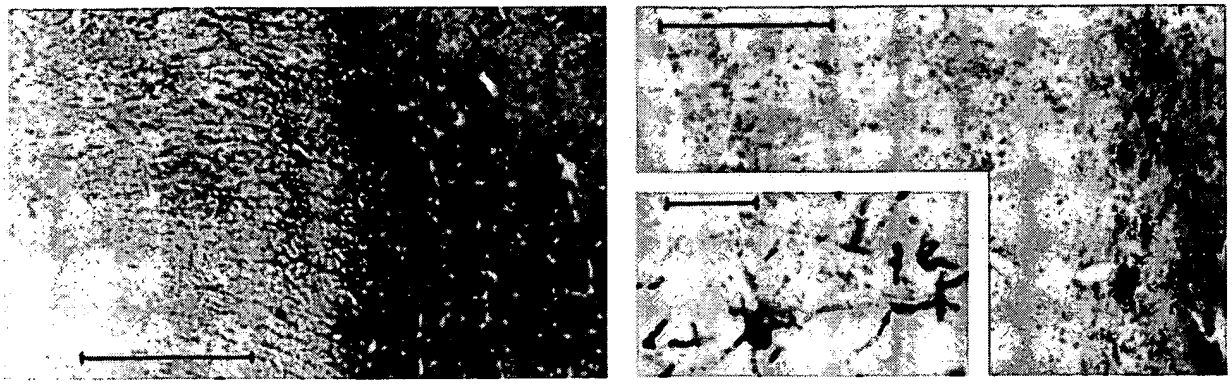
A



B

Fig 6. Immunocytochemistry for microtubule-associated protein 2. The neuronal architecture of the cortex appears normal (A), *but many subcortical neurons with random dendritic orientation are present* (B). *(Bars = 100 μm.)*

Fig 7. Immunocytochemistry for myelin proteins. Left panels (A, C, E, G) show normal brain; right panels (B, D, F, H) show Pelizaeus-Merzbacher disease. A and B show myelin basic protein (MBP); C and D show myelin-associated glycoprotein (MAG); E and F show 2',3'-cyclic nucleotide-3'-phosphodiesterase (CNP); and G and H show proteolipid protein (PLP) (antiserum to PLP apoprotein). The normal tissue shows intense myelin staining and the junction between cortex and white matter. The affected white matter shows islands of myelin-like sheaths with reactions for MBP, MAG, and CNP, but not with PLP. The insets reveal oligodendrocytes with delicate immunoreactive processes, which are connected to short segments of "myelin" (arrows). *(Bars = 1 mm; bars in insets = 50 μm. Insets were photographed with Nomarski optics.)*



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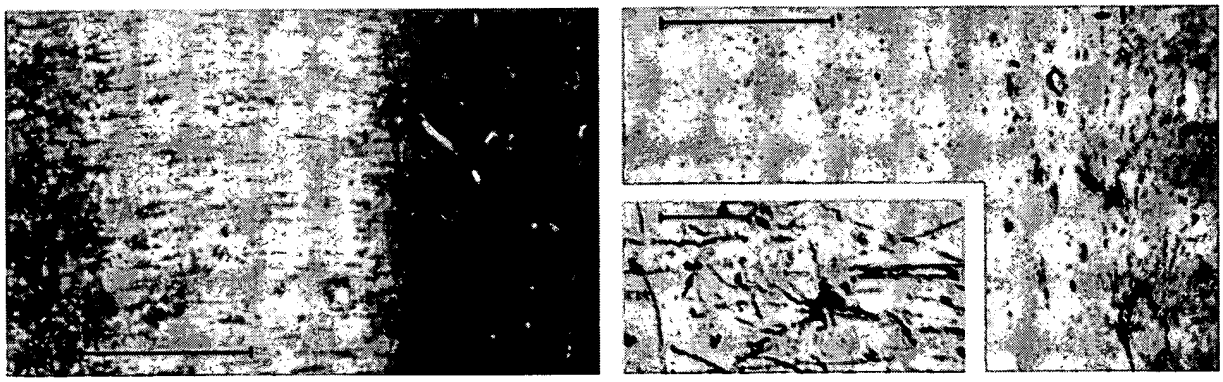

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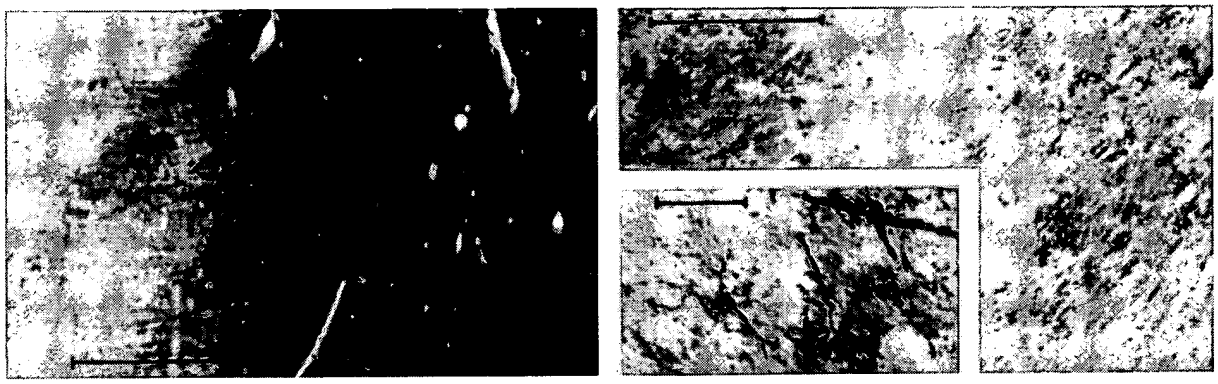

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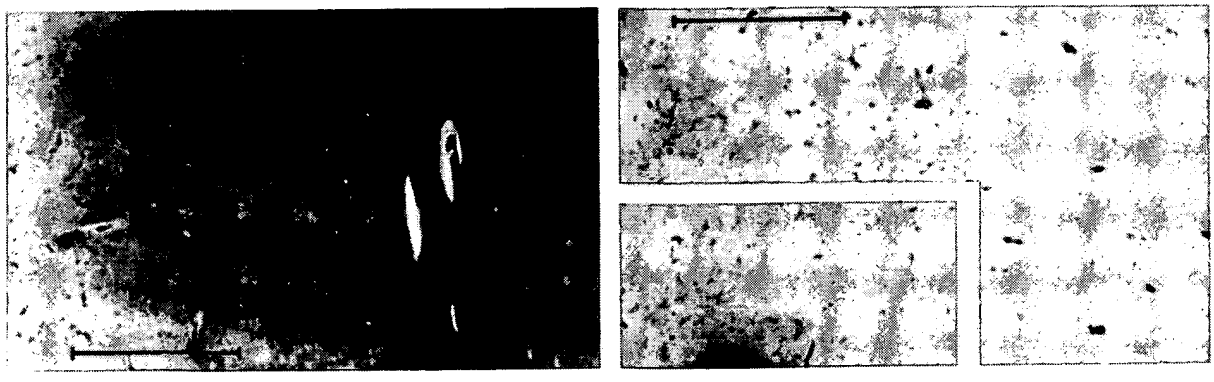

G H

Table 1. Biochemical Constituents in Brain Tissue from Pelizaeus-Merzbacher Disease Compared to Those in Normal Brain

Constituent[a]	Pelizaeus-Merzbacher Disease		Normal Control Subject	
	Gray Matter	White Matter	Gray Matter	White Matter
Water	86.600	88.800	82.400	70.500
Total protein	6.500	6.800	9.400	11.800
Total lipid	3.300	2.300	5.200	10.900
Triacylglycerol	0.256	0.148	0.527	1.186
Diacylglycerol (both isomers)	0.246	0.307	0.314	0.948
Monoacylglycerol (both isomers)	0.158	0.175	0.567	1.186
Galactolipids	0.862	0.422	6.500	27.500
Cerebrosides	0.504	0.289	4.320	15.810
Sulfatides	0.216	0.186	3.500	4.530
Phospholipids	31.420	21.680	29.350	65.900
Phosphatidylethanolamine	9.360	5.420	5.800	18.100
Phosphatidylserine and phosphatidylinositol	2.560	1.650	2.020	4.780
Phosphatidylcholine	12.480	7.310	3.160	12.700
Sphingomyelin	3.080	2.210	6.500	14.300
Cardiolipin	0.931	0.358	0.287	0.823
Cholesterol	21.400	15.200	26.900	65.900
Cholesteryl esters	1.230	1.080	0.980	1.870

[a]Results for water, total protein, and total lipid are expressed as the percentage of wet weight. Data for lipids are given as nanomoles per milligram wet weight.

The differences in the concentrations of neutral lipids are difficult to interpret because of the well-established postmortem changes in the acylglycerols. Nevertheless, their low concentration in the affected brain may reflect the lack of more complex precursor lipids such as the phospholipids.

SDS-PAGE is illustrated in Figures 8 and 9. Immunoblots revealed normal GFAP patterns, but a striking lack of MBP, MAG, and CNP (see Fig 8). Only the overloading of sample wells, as shown for MBP in lanes 6 and 7 of Figure 8, revealed a visible immunochemical reaction product. On immunoblots for CNP (see Fig 8), the extract of PM white matter showed an unidentified band (lane 12) due to a protein of higher molecular weight than either of the normal CNP bands (doublet) (lane 13). Its estimated molecular weight (54,000) corresponds to the protein detected in human oligodendroglioma by Sheedlo and co-workers [46] and Sprinkle and colleagues [50]. Extraction and purification of PLP [3] revealed only very small amounts of protein in the eluate from the Sephadex LH 60 column. Subsequent SDS-PAGE showed no detectable PLP apoprotein on the Coomassie blue–stained gels (Fig 9, lane 3) or on immunoblots (Fig 9, lane 4). In contrast, normal white matter gave abundant staining and immunochemical reaction product for PLP (apoprotein), including the main fraction (molecular weight, about 24,000), DM 20, higher-molecular-weight aggregates, and a faster band.

ELISA data for MBP, MAG, CNP, and PLP are compared in Table 2. CNP activity in normal white matter was 45.7 units/gm wet weight (5.5 units/mg protein). In affected white matter, the activity was 0.79 units/gm wet weight (0.065 units/mg protein) (1.7% of normal). Residual CNP immunoreactivity and enzyme activity thus were similar.

Discussion

Clinical Features and Genetics

In his extensive review of PM, Seitelberger [45] divided the reported cases into classic, connatal, transitional, adult, and atypical forms. Important criteria for this subclassification were age of onset, severity of illness, and the tigroid appearance on myelin stains of sections from white matter. The clinical features correlated quite well with the degree of myelin deficiency. In patients with early onset, the lack of myelin was especially severe, whereas in the "classic" cases myelin was better retained. This subdivision may become obsolete after the genetic defect is better characterized. Complete or nearly complete absence of PLP may be true for the connatal cases, and relative deficiency for cases with later onset and better function.

The disorder may be diagnosed during life if X-linkage can be established beyond doubt. Muscle atrophy and areflexia are misleading signs because neurologists tend to associate demyelinating diseases of the CNS with upper motor neuron signs. Nerve biopsies should be normal in PM, though electrodiagnosis may reveal denervation. A precise prenatal diagnosis is not yet available, but determining the sex of the un-

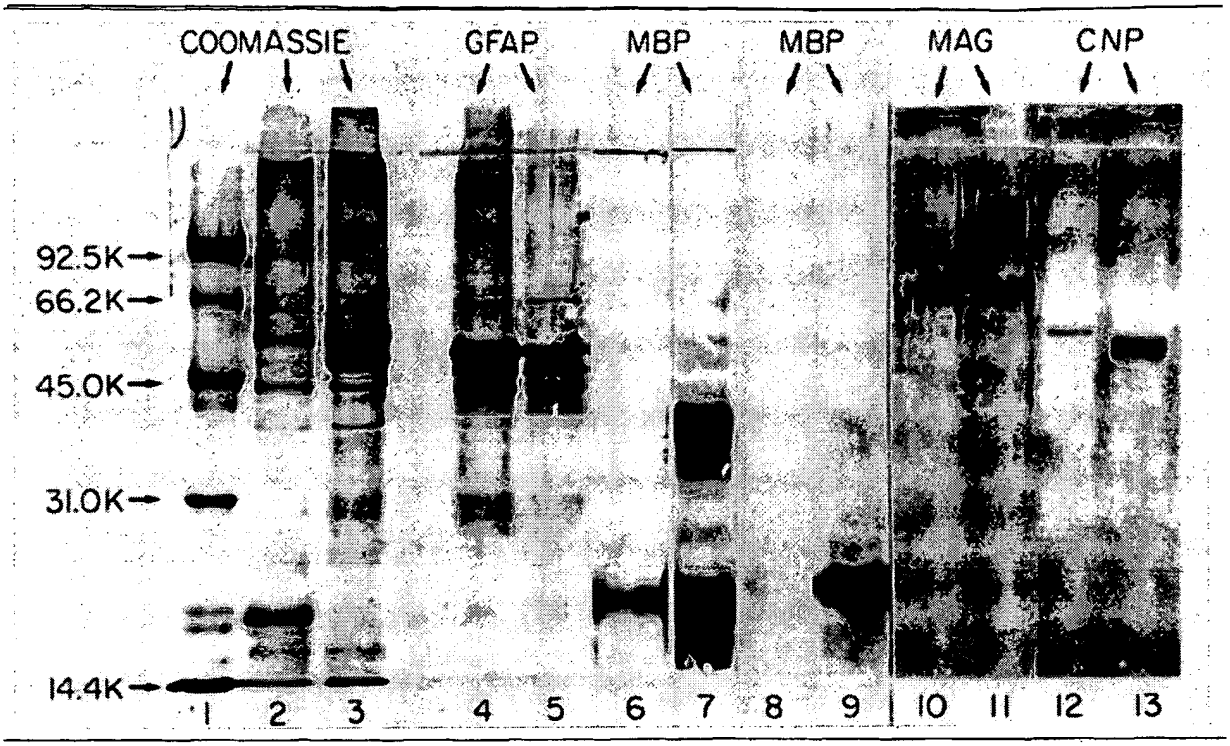

Fig 8. Sodium dodecylsulfate–polyacrylamide gel electrophoresis and immunoblots of glial fibrillary acidic protein (GFAP), myelin basic protein (MBP), myelin-associated glycoprotein (MAG), and 2',3'-cyclic nucleotide-3'-phosphodiesterase (CNP). Lanes 1 through 3 were stained with Coomassie Brilliant Blue G 250. Lanes 4 to 13 are immunoblots for the proteins indicated on the top. Lane 1: molecular weight standards; lanes 2, 5, 7, 9, 11 and 13: normal white matter; lanes 4, 6, 8, 10, and 12: white matter from the Pelizaeus-Merzbacher brain. Identical amounts of total protein were applied to the sample wells. The stained gel (lanes 2 and 3) shows the lack of MBP in the affected tissue (lane 3), which is further documented in lanes 6 and 8. Lanes 6 and 7 were deliberately overloaded to demonstrate the small residual amounts of MBP in the affected brain (lane 6). Lane 10 reveals the lack of MAG and dMAG but shows an unidentified band of 68 kD, which is also present in normal brain (lane 11). It is likely that this band is in part responsible for the high residual enzyme-linked immunosorbent assay value for MAG (Table 2). Lane 12 lacks the normal doublet of CNP but reveals an unidentified additional band that cross-reacts with anti-CNP [46, 50].

born infant may be offered to concerned parents of a potentially affected boy.

The presence of normal peripheral myelin that is devoid of immunoreactive PLP and X-linkage suggested that the genetic code for PLP lay in the human X chromosome, but proof of this assignment had to await the modern methods of molecular biology (see discussion following).

Pathogenesis of PM

Traditional stains for myelin sheaths revealed a tigroid pattern in the white matter of classic cases but total loss in the connatal cases [45]. Immunocytochemical stains for the myelin proteins MBP, MAG, and CNP in this study also revealed a tigroid collection of "myelin," so the distinction between the two types of the disorder more likely reflects severity rather than essential differences. The observations made here cannot settle the frequently heated argument on the nature of myelin loss, i.e., whether the disease represents failure to myelinate or demyelination. In cultured oligodendrocytes of rat fetuses and neonatal rats, galactocerebroside immunoreactivity can be detected by immunocytochemistry before the appearance of MBP, MAG, and PLP. MAG is the first immunoreactive protein, appearing about 10 days after birth. MBP and PLP are seen in comparable locations of the cell at about 15 days after birth [11]. While these observations may not be entirely applicable to the developmental time course in normal human brain or that of the patient with PM, we suggest that lack of PLP biosynthesis is responsible for the defective assembly of proper myelin sheaths. Under this assumption, PM may be viewed as a failure to myelinate. Lipids are not incorporated in adequate quantities (or not at all). The lack of myelin lipid, reported by several authors [1, 4, 56, 59] and in this paper, may be interpreted as a secondary phenomenon. Zeman and associates [63] were the first to suggest that the metabolic lesion did not lie with myelin lipids but with myelin proteins. They thought that the presence of PLP in the CNS and its absence from the PNS accounted for the confinement of PM to the brain and spinal cord.

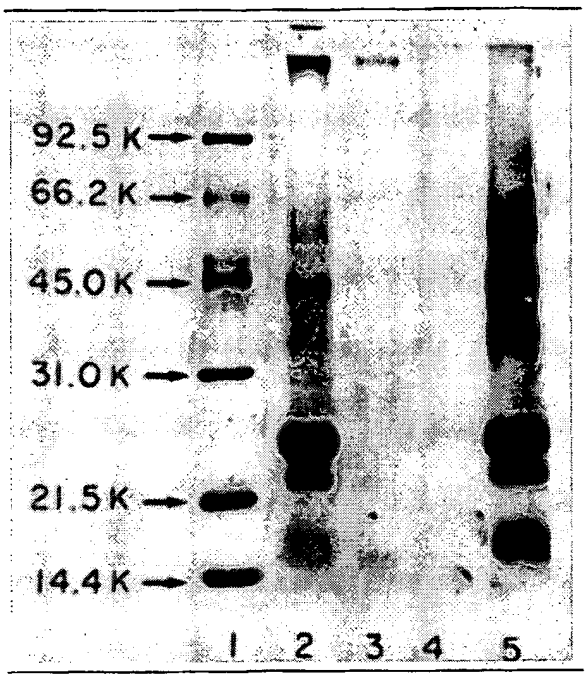

Fig 9. Sodium dodecylsulfate–polyacrylamide gel electrophoresis and immunoblots of proteolipid protein (PLP). Lanes 1 through 3 have been stained with Coomassie Brilliant Blue G 250; lanes 4 and 5 are immunoblots; lanes 2 and 5, normal white matter; and lanes 3 and 4, Pelizaeus-Merzbacher brain. Lanes 2 and 5 reveal the normal major PLP band and the faster moving DM 20 fraction. Aggregates of higher molecular weight and a band of about 18 kD are also present. Lanes 3 and 4 show the complete absence of PLP in the extract of the affected brain (antiserum to PLP-apoprotein).

Table 2. Comparison of MBP, MAG, CNP, and PLP in Normal White Matter and in White Matter of a Patient with Pelizaeus-Merzbacher Disease

Protein	Method	Pelizaeus-Merzbacher Disease[a]		Normal White Matter[b]
MBP	ELISA	0.105	(0.21)	7.0
MAG[c]	ELISA		(25.0)	
	Radioimmunoassay[d]		(2–6)	
	Immunoblot/densitometry[e]		(0.1)	
CNP[c]	ELISA		(0.2)	
PLP[f]	ELISA	0	(0)	8.0

[a]Milligram per gram wet weight; percentage of normal in parentheses.
[b]Milligram per gram wet weight.
[c]For MAG and CNP, protein standards were not available and only comparative analysis based on identical wet weight could be made.
[d]Performed by Dr Richard Quarles, as described in [25, 38].
[e]Performed by Drs Johanna Moller and Richard Quarles, as described in [61].
[f]For ELISA of PLP, the antiserum to PLP apoprotein was used.

MBP = myelin basic protein; MAG = myelin-associated glycoprotein; CNP = 2',3'-cyclic nucleotide 3'-phosphodiesterase; PLP = proteolipid protein; ELISA = enzyme-linked immunosorbent assay.

The lack of typical oligodendrocytes also implies a defect in cellular differentiation. In considering this lack of a specific cell type, "gliosis" in PM must be interpreted with some caution. It is more likely that the abundance of astrocytes in the affected white matter is due to lack of oligodendroglial differentiation rather than to gliosis in response to tissue destruction. While standard histological stains fail to reveal oligodendrocytes, immunocytochemical reactions for MBP, MAG, and CNP disclose cells with a few processes attached to fragmentary myelin–like sheaths. Anti-PLP fails to reveal similar cells. These cells showing abortive efforts at myelination may be interpreted as oligodendrocytes that lack the genetic information for the biosynthesis of PLP, and thus remain immature by biochemical and morphological criteria. Hartman and co-workers [18] examined the maturation of rat brain myelin by immunocytochemistry using a PLP-antiserum, and illustrated the appearance of immunoreactivity in cell bodies and processes of oligodendrocytes, and subsequent reactivity in myelin sheaths. As myelin became more and more abundant, the reaction product in the oligodendroglial processes disappeared. It is thus not surprising that oligodendrocytes were not stained in normal adult human brain tissue. Lack of staining of oligodendroglial processes in normal adult brain tissue was true also for MBP, MAG, and CNP (this study), although vibratome sections permitted visualization of their somas with anti-MBP [52]. The staining of cell body and delicate varicose processes shown in this paper (MPB, MAG, CNP) is strikingly similar to the differentiating oligodendrocytes observed in normal brain at an early age. Their presence in an adult patient with PM strongly supports an early arrest of differentiation, most likely due to lack of PLP. The concentration of this protein was determined only once before in the brain of a patient with PM [42] and was found to be reduced to about 25% of normal.

While PLP appeared to be totally absent, as determined by immunocytochemistry and ELISA, other myelin proteins were present, though in greatly reduced amounts. A possible explanation is lack of retention. Although MBP, MAG, and CNP are perhaps synthesized in amounts adequate for myelination, the lack of PLP leads to their removal rather than to incorporation into myelin.

Deficiency of PLP in the jimpy mouse [26] and myelin-deficient rat [60] has now been documented. Other myelin proteins are present in reduced amounts. Brain tissue of a myelin-deficient rat that we recently studied using an antiserum to MBP revealed similar islands of abortive myelination.

It is not clear why neuronal migration in PM is impaired. Severe heterotopia does not seem to occur in this disease, but the arrest of centrifugal migration

from the germinal matrix likely antedates the defective myelination.

Molecular Biology in X-Linked Myelin Deficiencies

Several investigators have performed in vitro translation experiments with messenger ribonucleic acid (mRNA) from the brains of jimpy mice [40, 48]. The biosynthesis of most myelin proteins in cell-free in vitro translation systems was reduced, but PLP content was clearly most seriously depressed. After the cloning of labeled cDNA probes [8, 9, 23, 34, 36], the amounts of PLP-mRNA could also be measured directly and were found to be greatly reduced [13, 15, 23]. The reduction depended on the postnatal age of the jimpy hemizygote and was most severe at 20 days or later. The residual major PLP-mRNA of the jimpy was also somewhat reduced in size (by about 100 base pairs) [13, 15]. In the myelin-deficient rat, mRNA for PLP and MBP was similarly reduced [62]. Willard and Riordan [58] used a cloned PLP-cDNA probe to map the human genome and found effective hybridization with the X chromosome and specifically with the Xq13-Xq22 segment of the long arm. The probe also detected the PLP gene in a comparable region of the mouse X chromosome, which includes the jimpy locus. Hoffman-Chudzik and colleagues [21] and Fahim and Riordan [13] reported that digestion of DNA from jimpy mice with restriction endonucleases did not reveal major rearrangements of the PLP gene. Fahim and Riordan [13] have now prepared DNA from white cells and brain tissue of 4 unrelated patients with PM and have found evidence of a PLP gene rearrangement in one. The mutation on the X chromosome thus remains elusive and includes the additional possibility of defective gene regulation. The shorter PLP-mRNA favors a defect in PLP gene structure. The human PLP gene has now been fully characterized [Hudson, Puckett, and Lazzarini, personal communication, 1986], offering a direct comparison with the PM gene.

Supported by the Veterans Administration and the Department of Human Genetics of Hamburg University, Hamburg, West Germany. Antisera were supplied by Drs Marjorie B. Lees (PLP), Robert M. Herndon (MBP), Marian W. Kies (MBP), Nancy Sternberger (MAG), Richard H. Quarles (MAG), Terry Joe Sprinkle (CNP), Richard B. Vallee (MAP 2), and Donald E. Schmechel (human NNE). Dr Marian Kies and Mrs Gladys Deibler supplied purified bovine MBP. Dr Robert A. Lazzarini donated the rabbit polyclonal antiserum to synthetic PLP-peptide. Dr Ingrid Willers maintains the fibroblast cultures at the Department of Human Genetics of Hamburg University. Lymphoblast cultures of the 2 patients in Figure 1 of this report and of the 3 female carriers are being maintained at the National Institute of General Medical Sciences Human Genetic Mutant Cell Repository, Institute for Medical Research, Camden, NJ 08103.

Presented in part before the 13th World Congress of Neurology, Hamburg, West Germany, September 1985.

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